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PATENT SPECIFICATION

DRAWINGS ATTACHED

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Date of Application and filing Complete Specification June 17, 1965.

No. 25705/56.

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Index at acceptance:—C6 F1; B1 X6

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COMPLETE SPECIFICATION

Apparatus for Culturing Microorganisms

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SPECIFICATION No. 1,037,759

Page 1, Heading, Date of Application and filing Complete Specification June 17, 1965. *for* "No. 25705/56" *read* "No. 25705/65"

THE PATENT OFFICE
20th March 1967

tinue the growth of the microorganisms it is necessary to inoculate fresh units of culture medium. Because of this, the culturing of such microorganisms presents recognised difficulties and disadvantages. Thus, the failure to inoculate fresh culture medium units will result in the loss of certain stock cultures. In order to inoculate fresh culture medium units, laboratories require the attention and attendance of highly skilled technicians, the use of elaborate sterilisation apparatus and special care in each transfer of the culture in order to prevent contamination.

It is an object of the present invention to overcome the difficulties and disadvantages heretofore encountered and to provide an improved apparatus in which the culturing of microorganisms may be carried out in a continuous fashion or over a prolonged period of time without the necessity of repeatedly inoculating fresh culture medium units.

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the microorganism culture. When the inner, tubular envelope is arranged to form the chamber for microorganisms in a suitable liquid and the outer envelope forms the chamber for the culture medium, I preferably provide the inner, tubular envelope with an openable and closeable inlet opening at one end and an outlet opening at the other end, these openings communicating with the inner envelope through the outer envelope.

An example of apparatus constructed in accordance with the invention is illustrated in the accompanying drawings in which:—

Figure 1 is an elevation of the culturing apparatus, and

Figure 2 is a cross-section as seen in the direction of the arrows on the line 2—2 of Figure 1.

The illustrated apparatus comprises generally an outer envelope 10 enclosing a first liquid chamber and an inner envelope 12

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COMPLETE SPECIFICATION

Apparatus for Culturing Microorganisms

I, JOHN HANNA BREWER, a citizen of the United States of America, residing at 425 Oak Lane, Towson, County of Baltimore, Maryland, United States of America, do hereby declare the invention, for which I pray that a patent may be granted to me, and the method by which it is to be performed, to be particularly described in and by the following statement:—

This invention relates to apparatus for culturing of microorganisms such as bacteria, yeast and moulds and particularly for the prolonged or continuous culturing of such microorganisms.

It is a recognised phenomenon that when microorganisms such as bacteria, yeast and moulds are cultered in a unit of culture medium, the microorganisms go through a cycle of growth and development involving an initial accelerated growth until a peak is reached followed by a rapid decline. At this stage of the development in order to continue the growth of the microorganisms it is necessary to inoculate fresh units of culture medium. Because of this, the culturing of such microorganisms presents recognised difficulties and disadvantages. Thus, the failure to inoculate fresh culture medium units will result in the loss of certain stock cultures. In order to inoculate fresh culture medium units, laboratories require the attention and attendance of highly skilled technicians, the use of elaborate sterilisation apparatus and special care in each transfer of the culture in order to prevent contamination.

It is an object of the present invention to overcome the difficulties and disadvantages heretofore encountered and to provide an improved apparatus in which the culturing of microorganisms may be carried out in a continuous fashion or over a prolonged period of time without the necessity of repeatedly inoculating fresh culture medium units.

According to my invention, such apparatus comprises a closed outer envelope forming a first liquid chamber and an inner envelope formed of an elongated semi-permeable tubular membrane disposed inside the outer envelope and forming a second liquid chamber, one of the liquid chambers serving as a culture medium chamber and the other liquid chamber serving as a chamber for microorganisms in a suitable liquid whereby nutrients from the culture medium can dialyse through the membrane to the liquid containing the microorganisms and waste products from the microorganisms can diayse through the membrane to the culture medium, the envelope for the culture medium chamber being produced with openable and closable remotely spaced inlet and outlet openings so that culture media can be passed through the chamber.

The toxins produced by the microorganisms do not dialyse through the semi-permeable membrane but remain in the chamber with the microorganism culture. When the inner, tubular envelope is arranged to form the chamber for microorganisms in a suitable liquid and the outer envelope forms the chamber for the culture medium, I preferably provide the inner, tubular envelope with an openable and closeable inlet opening at one end and an outlet opening at the other end, these openings communicating with the inner envelope through the outer envelope.

An example of apparatus constructed in accordance with the invention is illustrated in the accompanying drawings in which:—

Figure 1 is an elevation of the culturing apparatus, and

Figure 2 is a cross-section as seen in the direction of the arrows on the line 2—2 of Figure 1.

The illustrated apparatus comprises generally an outer envelope 10 enclosing a first liquid chamber and an inner envelope 12

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disposed in said first liquid chamber and enclosing a second liquid chamber. The outer envelope is made of flexible plastics material impervious to liquids and inert to the culture medium to be used and to the microorganisms to be cultured. It comprises two layers of plastics material 14 and 16 heat sealed around the edges as shown at 18 to provide a liquid-tight chamber.

I find that examples of suitable plastics materials forming the outer envelope are polyethylene, polyvinylchloride and the copolymers thereof, polypropylene, tetrafluoroethylene and tetrafluorochloroethylene. Where the microorganisms or bacteria are anaerobic, the plastics material should be impervious to air. Where the microorganisms or bacteria being cultured are aerobic, then the plastics material should be pervious to air while at the same time impervious to the liquid contained therein.

Thin sheets of polyethylene and polypropylene of the order of .002 inches thick will transmit oxygen and are suitable for use with aerobic microorganisms. In this connection, for the deep culturing of aerobic microorganisms both layers of the envelope should be made of an air pervious plastic, while for the pellicle growth of aerobic microorganisms one layer should be air pervious and the other layer should be air impervious.

Thicker sheets of all the above-indicated plastic materials of the order of .006 inches or more in thickness are air impervious. Similarly, laminated layers of thinner sheets are also air impervious. For the culturing of anaerobic microorganisms both layers of the envelope should be air impervious.

The inner envelope 12 is in the form of an elongated tubular synthetic or natural semi-permeable membrane. For this purpose, I may use natural sausage casings made from the intestines of hogs, sheep or other animals, synthetic sausage casings made of regenerated cellulose such as the synthetic sausage casing made and sold by Visking Company under the Registered Trade Mark "Visking". I may also make the tubular inner envelope from any other natural or synthetic semi-permeable membrane material suitable for dialysis, such as parchment, collodion or cellulose acetate. The inner envelope is of serpentine configuration.

It will be seen that both envelopes have remotely disposed inlet and outlet openings. Thus, the outer envelope 10 has inlet and outlet openings 20 and 22 which may have cut off valves for controlling the introduction and withdrawal of liquid. Similarly, the inner envelope has inlet and outlet openings 24 and 26 which extend through and are sealed in the outer envelope as shown. The outlets 24 and 26 may also be provided with suitable control valves.

In using my improved apparatus, I place the culture medium in one of the envelopes and I place suitable liquid into which the microorganisms may be introduced in the other envelope. A suitable liquid for this purpose is either distilled water, saline, solutions of other salts such as sodium citrate and also culture medium. Both the culture medium and the liquid should be sterilised either before or after introduction into the envelopes. Thereafter, the desired microorganisms are introduced into the distilled water or saline. Nutrients from the culture medium will dialyse through the semi-permeable membrane into the liquid containing the microorganisms. In addition, waste products from the microorganisms will dialyse through the semi-permeable membrane into the culture medium. On the other hand, I have found that toxins produced by the microorganisms will not dialyse through the semi-permeable membrane but will remain in the liquid with the microorganisms. In due course, after the microorganisms have grown and developed, new culture medium can be introduced at a relatively slow rate through the inlet opening of the envelope containing the culture medium and old culture medium containing waste products can be withdrawn at the same rate through the outlet opening. This ensures a supply of fresh nutrient for the microorganisms and also prevents an undue concentration of waste products. The microorganisms in the envelope containing the distilled water or saline can be cultured on a batch basis or on a continuous basis. When cultured on a batch basis, the contents of the envelope containing the microorganisms can be withdrawn when a maximum growth has been obtained which, it has been found, far exceeds the maximum growth obtained under normal conditions with a similar volume of liquid and nutrient. If it is desired to culture the microorganisms on a continuous basis after an optimum growth has been achieved, the liquid having microorganisms therein can be withdrawn from the envelope containing them at a slow rate and a corresponding quantity of fresh liquid can be aseptically introduced throughout the inlet opening.

I have found that by improved apparatus is particularly useful for the production of toxins for use in making anti-toxins. As previously indicated, the toxins do no dialyse through the semi-permeable membrane but remain in the liquid with the microorganisms. Accordingly, after the liquid containing the microorganisms is withdrawn from the envelope either on a continuous or batch basis the toxins can be separated from the microorganisms in the usual manner.

In using my apparatus for any of the purposes indicated above, the culture medium containing the nutrients may be placed in the outer envelope and the microorganisms placed in the inner envelope or, if desired, the

relationship may be reversed with the culture medium in the inner envelope and the liquid and microorganisms in the outer envelope.

Any suitable culture medium containing nutrients which will dialyse through the semi-permeable membrane may be employed, such as plain or dextrose broth, brain, meat, and gelatin or a synthetic medium containing amino acids of arginine, histidine, tyrosine, valine, leucine, isoleucine, and tryptophan, vitamins, such as riboflavin, panthothenic acid, thiamine, folic acid, biotin, pyridoxine and nicotinic acid, adenine, uracil and oleic acid. Also many different types of micro-organisms may be cultured in by apparatus, as for instance anaerobic microorganisms such as *Clostridium tetani, Clostridium septicum, Clostridium welchii, Clostridium novyl, Clostridium histolyticum, Clostridium sporogenes, Clostridium chavel, Clostridium botulinum,* streptococci, or aerobic microorganisms such as *Corynebacterium diphtheriae, Bacillus anthracis, Vibrio cholerae, Staphylococcus aureus* and *Staphylococcus citreus.*

The following are specific examples of my invention:

Example 1

Anaerobic Growth of *Clostridium tetani*: An envelope of the type shown in Figures 1 and 2 is made of polypropylene plastic material (.006 in. thick), and has the following dimensions: unit length 14 in., unit height 17 in., unit width 2 in.

The semi-permeable membrane disposed within the envelope as shown in Figures 1 and 2 is a synthetic sausage casing sold under the Registered Trade Mark "Visking". The envelope is fitted with inlet and outlet openings similar to the openings as shown in Figures 1 and 2 for the envelope.

The culture medium used for the growth of *Clostridium tetani* has the following composition: arginine, histidine, tyrosine, valine, leucine, isoleucine, trytophan, riboflavin, pantothenic acid, thiamine, folic acid, biotin, pyridoxine, nicotinic acid, adenine, uracil and aleic acid. The medium is sterilised by filtration through a Seitz filter, de-aerated, placed in the envelope and the inlet opening closed. The physiological saline solution is sterilised by autoclaving at 121°C., for twenty minutes, de-aerated and placed in the semi-permeable membrane through the inlet opening.

Clostridium tetani is added to the physiological saline solution before the inlet opening is closed. The envelope is placed in a suitable thermal environment (37°C.) until growth of the *Clostridium tetani* reaches its maximum point.

The nutrients of the culture medium dialyse through the semi-permeable membrane, and the waste products of *Clostridium tetani* dialyse through the semi-permeable membrane into the culture medium. The toxins that are produced by the *Clostridium tetani* are selectively retained in the saline solution and do not dialyse through the semi-permeable membrane. The culture medium is withdrawn from the envelope from the outlet opening at the same rate as new medium is being added at the inlet opening, thus reconstituting new medium for the medium containing the waste products of the *Clostridium tetani* which will allow additional growth of the organism. New physiological saline solution is added to the semi-permeable membrane through its inlet opening, and at the same time the saline solution containing the microorganisms and toxins of the *Clostridium tetani* are withdrawn. The bacteria is separated from the toxins in accordance with standard laboratory procedures.

Thus, I have shown a method for continuous preparation of toxins for production of *Clostridium tetani* toxoid.

Example 2

The same procedure is followed as in Example 1 except that the culture medium is placed within the semi-permeable membrane and the physiological saline solution is placed in the envelope. The inoculation of the *Clostridium tetani* may either be added to the saline solution when the inlet opening has not yet been sealed, or may be injected through the envelope using a hypodermic syringe and needle. Upon withdrawal of the needle, a suitable sterile pressure sensitive adhesive tape may be used to close the hole caused by the puncture.

Example 3

Aerobic Growth of *Corynebacterium diptheriae*: An envelope of the type shown in Figures 1 and 2 is made of polypropylene plastic material (.002 in. thick) which will transmit oxygen or air but which is impervious to the solution contained therein. The other side of the envelope is air or oxygen impervious and .008 in. thick. The assembly of the envelope and semi-permeability membrane are the same as in Example 1. The culture medium for the grown of *Corynebacterium diphtheriae* used has the composition: extract of fresh lean beef 400 to 600 g., peptone 5 g., NaCl 5 g., and the addition of distilled water to a resultant volume of 1,000 ml. The pH is adjusted from 7.8 to 8.0. The solution is heated to 100°C., for 20 minutes to coagulate tissue proteins from the fresh lean beef, and these are removed by filtration. The medium is placed in the envelope and physiological saline solution is placed in the semi-permeable membrane and inoculated with *Corynebacterium diphtheriae* before sealing the inlet opening with a cotton plug. The envelope is suspended in a thermal atmosphere of 36° to 37° C. with the air permeable layer exposed to the air until pellicle growth develops on the inner surface of the semi-

permeable membrane exposed to the air permeable layer. The nutrients of the culture medium dialyse through the semi-permeable membrane into the saline solution and the waste products of *Corynebacterium diphtheriae* dialyse through the semi-permeable membrane from the saline solution to the culture medium. The maximum toxin production occurs from 7 to 10 days after the inoculation, at which time new culture medium is added at the inlet opening at the same rate as the culture medium containing the waste products of *Corynebacterium diphtheriae* is withdrawn. Fresh saline solution is added to the semi-permeable membrane through the inlet opening at the same rate as the saline solution containing the toxin and diphtheria bacillus are withdrawn. The bacteria are separated from the toxins in accordance with standard laboratory procedures.

Thus, I have shown a method for the continuous preparation of toxins or aerobic bacteria, said toxin may be used for the production of *Corynebacterium diphtheriae* toxoid.

EXAMPLE 4

Aerobic Growth and Sperulation of *Bacillus Stearothermophilus*:

An envelope of the type described in Example 3 is used and its assembly is the same as that used in Example 1.

The culture medium for the growth of *Bacillus stearothermophilus* and subsequent spore production has the following composition: casein peptone 5 gm., plant peptone 3 gm., beef extract 5 gm. and the addition to distilled water to a resultant volume of 1,000 ml. The pH is adjusted to 7.5. The solution is sterilised by autoclaving at 121°C. for 20 minutes. The sterile medium is placed in the envelope and sterile physiological saline is placed in the semi-permeable membrane and inoculated with *Bacillus stearothermophilus* before sealing the inlet opening with a cotton plug. The envelope is suspended in a thermal atmosphere of 60° to 63°C. with the air permeable layer exposed to the air until maximum growth develops. The nutrients of the culture medium dialyse through the semi-permeable membrane into the saline solution and the waste products of *Bacillus stearothermophilus* dialyse from the semi-permeable membrane containing the saline solution to the culture medium. Maximum growth and production of spores occur within 24 to 48 hours after inoculation, at which time new culture medium is added at the inlet opening at the same rate as the culture medium containing the waste products of *Bacillus stearothermophilus* is withdrawn. Fresh saline solution is added to the semi-permeable membrane through the inlet opening at the same rate as the saline solution containing the spores and stearothermophilus bacillus is withdrawn. The spores are separated from the non-spores of *Bacillus stearothermophilus* and other cellular debris by centrifugation and washing in accordance with standard laboratory procedures.

Thus, I have shown a continuous preparation of spores and aerobic bacteria, said spores may be used as a viable biological system for measuring heat sterilisation.

WHAT I CLAIM IS:—

1. Apparatus for the prolonged or continuous culturing of microorganisms comprising a closed outer envelope forming a first liquid chamber and an inner envelope formed of an elongated semi permeable tubular membrane disposed inside the outer envelope and forming a second liquid chamber, one of the liquid chambers serving as a culture medium chamber and the other liquid chamber serving as a chamber for microorganisms in a suitable liquid whereby nutrients from the culture medium can dialyse through the membrane to the liquid containing microorganisms and waste products from the microorganisms can dialyse through the membrane to the culture medium, the envelope for the culture medium chamber being provided with openable and closeable remotely spaced inlet and outlet openings so that culture medium can be passed through the chamber.

2. Apparatus according to claim 1, in which the outer envelope includes an air pervious portion so that aerobic microorganisms can be cultured.

3. Apparatus according to claim 1 or claim 2 in which the outer envelope is made of liquid impervious flexible plastics material.

4. Apparatus according to any one of the preceding claims in which the inner, tubular envelope is arranged to form the chamber for microorganisms in a suitable liquid and the outer envelope forms the chamber for the culture medium, the inner, tubular envelope having an openable and closeable inlet opening at one end and an outlet opening at the other end, these openings communicating with the inner envelope through the outer envelope.

5. Apparatus according to any one of the preceding claims, in which the inner tubular envelope is of serpentine configuration.

6. Apparatus according to claim 1, constructed substantially as hereinbefore described with reference to the accompanying drawings.

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1,037,759 COMPLETE SPECIFICATION

1 SHEET

This drawing is a reproduction of the Original on a reduced scale.

FIG. 1

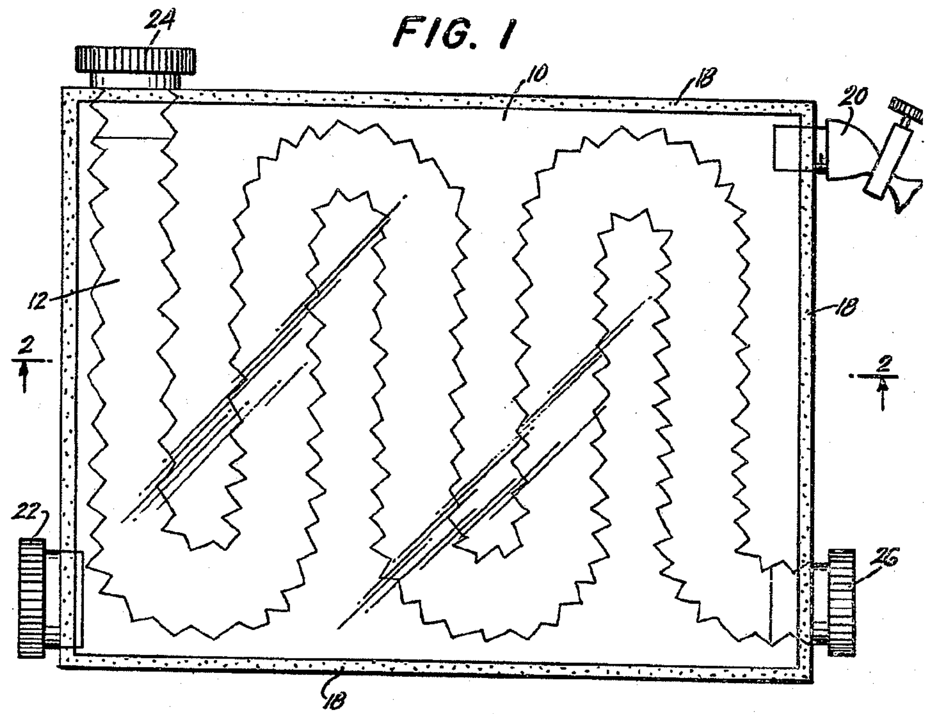

FIG. 2